Identifying genes that interact with calcineurin during egg activation in *Drosophila melanogaster*

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ABSTRACT

When an egg is fertilized, before the new embryo can begin to develop, the egg must be "activated." The process of activation triggers the egg to complete meiosis, to begin translating new proteins, to degrade proteins and RNAs that are no longer required, and to modify its membranes to form a harder outer covering. The universal signaling event is an increase in calcium levels that occurs in the egg at this time. In many cell types, calcium causes changes through a conserved signaling pathway by binding to a protein called calmodulin, which in turn regulates the activities of other proteins.

Calcineurin, a phosphatase, is one of those other proteins. It has been proven experimentally that calcineurin plays a critical role in regulating egg activation in fruit flies and in frogs. For example, *Drosophila* females expressing a constitutively active calcineurin (*cnA-act*) in their germline are sterile. To identify the proteins in the pathway by which calcineurin regulates egg activation, the Toshiro Aigaki lab tested whether there were *Drosophila* genes that interact genetically with calcineurin mutations. To do this, they searched for chromosomal regions whose deletion suppresses the sterility of *cnA-act* female flies. Fourteen large chromosomal regions were identified.

To define the precise gene(s) within each region that suppresses the sterility of *cnA-act* females, I have tested smaller deletions, and then individual mutations, within the five regions I am examining for interaction with *cnA-act*. For three of the deletions, I have identified genes whose mutants significantly restore fertility of *cnA-act* females, and thus is a candidate to act within the calcineurin pathway. The first is *CG6927*, a gene that is expressed in the ovary but currently has no known function. The second is *eIF3-S8*, a transcription initiation factor. The third is *RpS24*, a ribosomal protein. The fourth is *CG42565*, which like *CG6927*, has no known function. In the other two regions, I have narrowed down the *cnA-act* interacting genes to a small number of candidates, instead of the hundreds of genes within the original large region. I am currently testing the remaining candidates.

INTRODUCTION

EGG ACTIVATION

Egg activation is the transition of an egg from a mature oocyte to a state that can undertake embryo development. During egg activation, several key processes occur: the eggshell is modified to prevent polyspermy and to protect the future embryo, meiosis resumes and completes, translation of new proteins initiates from stored maternal mRNAs, while other maternal mRNAs are degraded, and several proteins get dephosphorylated (Horner & Wolfner, 2008a; Figure 1). In addition, the localization of certain mRNAs changes during this time (Weil et al., 2008). In fruit flies, egg activation is triggered by mechanical stimulation of the egg during ovulation. Fertilization is not required for activation (Heifetz et al., 2001; Horner & Wolfner, 2008b). *Drosophila* eggs can be activated mechanically, but they only complete meiosis and then stop developing because haploid zygotes are inviable (Doane, 1960). Prior to activation, *Drosophila* eggs are arrested in metaphase I of meiosis (King, 1970). It remains in this dormant state until it is released by egg activation.

CALCIUM SIGNAL TRANSDUCTION PATHWAY

Currently, the key fact known about the egg activation cell signal pathway is that there is a surge of calcium ions that activates a protein known as calmodulin (Klee et al, 1998). This surge of calcium is conserved in vertebrates and marine invertebrates (Stricker, 1999). Genetic and physiological evidence suggests that this calcium surge is conserved in *Drosophila* also (Horner et al., 2006; Horner & Wolfner, 2008a). The likely target of the increased calcium, calmodulin, positively regulates two downstream

proteins: calmodulin-dependent kinase II (*CaMKII*) and the phosphatase, calcineurin (Figure 1). In *Xenopus laevis*, upon activation, the influx of calcium triggers the release of the egg from meiotic arrest through the action of *CaMKII* (Liu & Maller, 2005). *CaMKII* phosphorylates ERP1, marking it for ubiquitination and subsequent degradation (Mochida & Hunt, 2007). This allows the resumption of the cell cycle since ERP1 is an inhibitor of the anaphase promoting complex/cyclosome (APC/C) so it maintains the egg in the arrested state prior to degradation (Rauh et al., 2005; Reber et al., 2008). Mutations in the γ isoform of *CaMKII* in mice cause female sterility since the eggs remain arrested and are never able to become activated (Backs et al., 2010).

Calcineurin was found to be activated by calmodulin during egg activation in *Xenopus laevis* (Nishiyama et al, 2007). The rise in intracellular calcium leads to the transient activation of calcineurin by the calcium-dependent calmodulin (Nishiyama et al., 2007). Calcineurin activation must then be inhibited in order for the cell to continue meiosis from where it had been arrested. This was proven by the fact that maintaining a high level of calcineurin after activation prevents the "growth of sperm asters" and hinders the "migration of male and female pronuclei towards each other . . . in fertilized eggs" (Nishiyama et al., 2007). Therefore, calcineurin must be properly regulated in order for continued embryonic development after fertilization. In 2006, Horner et al. and Takeo et al. found that mutations in *sarah* (*sra*), which encodes calcipressin, a regulator of calcineurin activity, result in female sterility due to failure of eggs to activate. Therefore, calcineurin is clearly an important component of the egg activation pathway, yet its actual role and specific gene targets remain unknown.

To further study the role of calcineurin, Toshiro Aigaki's lab created a constitutively active calcineurin (*cnA-act*) *Drosophila* mutant and generated fly lines in which this was expressed. These flies produced a form of calcineurin that is truncated at the catalytic C-terminus, preventing the regulatory subunit from being able to bind and inhibit the phosphatase activity. Females expressing *cnA-act* were sterile, with laying eggs that remained arrested in meiosis, suggesting that constitutive (rather than transient) activation of calcineurin interfered with egg activation (Takeo et al., 2010). Takeo et al. further found that certain deletions, when present heterozygously in *cnA-act* flies, counteract the effect of *cnA-act*, and restore fertility (Takeo et al., 2006; T. Aigaki, personal communication; Figure 2). I hypothesize that these deletions restored fertility by each removing a gene coding for a protein that interacts with calcineurin in the egg activation pathway. In deletion heterozygotes, only half the normal amount of their protein would be made due to the decreased gene dosage. If the protein were one through which calcineurin caused egg activation, lower doses of it should counter the effects of increased calcineurin. For example, lower levels of a protein with a similar function could mean that the excess calcineurin could take over its role and restore normal development. Another possibility is that, in the heterozygous mutants, there are lower levels of the protein of interest so the fact that calcineurin is constantly positively regulating it would not matter because there is only so much that can get activated regardless of how much active calcineurin is present. Finally, lower levels of this protein could release another protein from either being sequestered or inactivated so it can interact with calcineurin to inhibit its activity and restore normal levels.

RELEVANCE

This research is relevant scientifically and has potential to be of relevance to medical diagnostics. First, it will reveal a great deal about the calcineurin pathway, a conserved pathway in all organisms tested, and one that appears to be conserved for a role in activation. Despite its importance, many molecules that calcineurin regulates to cause egg activation are still unknown in all systems. The genetics method just described can provide a way to find these downstream molecules so that their actions and interactions can be studied.

Diagnostically, this research may become applicable in the future because the egg activation pathway in *Drosophila* is a model for that in mammals – even humans. Though the initial signal differs (sperm entry in mammals including humans, mechanosensitive triggers in *Drosophila*), the events triggered by the signal appear to be common to all. It is possible that some women may struggle with infertility due to the fact that the egg gets fertilized by the sperm but, for some unknown reason, does not continue to develop. If the proteins involved with egg activation can be identified and the pathway elucidated in a model organism like *Drosophila*, more can be understood about the mechanism in humans as well. This knowledge could, therefore, be applied in the future to designing diagnostic tests and possibly treatments for infertility in women.

OBJECTIVE

The overall purpose of my project was to define the precise gene(s) within each region identified that suppresses the sterility of *cnA-act* female and, therefore, act(s) within the calcium-triggered signal transduction pathway. The overall method was to

cross flies, so as to generate females carrying *cnA-act* and heterozygous for a mutant allele for each single gene uncovered by an interacting deficiency. The number of progeny produced by these females was compared to the number produced by *cnA-act* females. Genes of interest were ones whose mutant suppressed *cnA-act* and thus, resulted in higher fertility than the near-sterility of *cnA-act* females.

MATERIALS AND METHODS

FLY CULTURE

Fly stocks were raised on yeast-glucose-agar medium at 22.5± 2°C in a 12-h L:12-h D photoperiod. The wild-type used was the P2 strain of Oregon R (ORP2) (Allis et. al., 1977). The mutant stocks of genes of interest that were available were obtained from the Bloomington Drosophila Stock Center at the Department of Biology of Indiana University. Stocks were transferred to new vials every week to ensure optimal growth and to prevent propagation of mites.

GENETIC CROSS SCHEME

In order to examine the interaction between a gene of interest and calcineurin, it was necessary to obtain flies that carry both the *cnA-act* transgene construct with a truncated form of the C-terminal part of the protein and a P-element insertion in the gene of interest. Truncating the C-terminus, or catalytic subunit, prevents binding of the regulatory subunit, thereby rendering the protein constitutively active. To acquire these flies, *Drosophila* virgin females with mutations in individual genes of interest were crossed with males carrying *cnA-act,* driven in the germline by a *nanos* (*nos*) driver, over

a balancer (Figure 3). The Aigaki lab gave us a balanced stock of *cnA-act*/*TM3* flies for the purpose of this experiment (Takeo et al., 2006). The balancer used contained the markers *Stubble* and *Serrated.* Since heterozygous *cnA-Act* females are sterile, the *cnA-act* males must be regenerated with a new cross each generation. To do this, *w[1118]* females that carry the *TM3* (*Sb*, *Ser*) balancer must be crossed to the *cnA-Act* balanced males. The *cnA-Act/TM3* males generated from this cross are then crossed to females carrying a P-element insertion in the gene of interest (from a homozygous stock or from a balanced stock if the mutation was homozygous lethal; Figure 3). From the progeny, I collected non-*Stubble*, non-*Serrated* flies, which have constitutively active calcineurin and contain one copy of the mutant allele in the gene of interest. The control *cnA-Act* females used were from the cross that is used to regenerate the *cnA-Act/TM3* stock.

FERTILITY/FECUNDITY ASSAY

Experimental and control females were aged, as virgins, for 3-5 days with yeast and were then mated to wild-type (ORP2) males. Typically, 8-10 females were used per gene of interest (in some cases, fewer were used due to fitness of strain). After mating, the male was removed from the vial to prevent remating. After 24 hours, the females were transferred to new vials and the eggs they had laid in the first vial were counted. This was repeated for a total of five days. After two weeks, the progeny from each vial were counted. The hatchability ratio was calculated by dividing the number of progeny eclosed from a given vial by the total number of eggs that had been laid in that vial. I ran a Wilcoxon Test between the hatchability ratios of females carrying both the mutation of

interest and the *cnA-act* construct and those of females with just the *cnA-act* construct. P-values < 0.01 were considered significant.

RESULTS

Fertility/fecundity assays were used to analyze the potential interaction between calcineurin and candidates for the calcium-dependent egg activation pathway. This was done by crossing virgin females that are mutant for a candidate gene to *cnA-act* males (Figure 3). Genes were examined within several deletions that had been found to suppress sterility in a screen by the Aigaki lab.

The first deletion I examined, *Df(1)JC70*, is located on the X chromosome and was found to restore fertility of *cnA-act* females to 70.6%, meaning that 70.6% percent of eggs laid develop into adult flies (T. Aigaki, personal communication). I was able to reproduce this suppression of sterility result as well. I found that *Df(1)JC70* restored fertility of *cnA-act* females to an average of 82.0% (Figure 4). This deletion's high level of suppression and the fact that there were fewer candidate genes under it to screen gave me a greater chance of finding a gene that interacts in the calcineurin pathway. Since calcineurin is a phosphatase, it was initially believed that a deletion could have restored fertility by having removed a gene that coded for another phosphatase, a kinase or kinase binding protein, etc. Therefore, I first tested individual mutants with P-element insertions in genes coding for either kinases, phosphatases, or kinase-binding proteins uncovered by *Df(1)JC70*. None of the genes that I tested restored fertility to *cnA-act* females. As a result, 29 genes were eliminated as possible components of the egg activation signal transduction pathway, as there was no significant increase in hatchability ratios of virgin

females containing both a mutation in the gene of interest and the *cnA-act* construct compared with that of virgin females with only the *cnA-act* construct. Therefore, they will not be further discussed (Table 1). One gene, *CG6927*, was found to significantly suppress sterility when compared to *cnA-act* control females ($P<0.0001$; Figure 5).

Other deficiencies found in the Aigaki screen to suppress sterility of *cnA-act* females span many more genes than *Df(1)JC70*. Therefore, to narrow down the number of candidate genes, I performed fertility/fecundity assays on smaller deletions within each of 4 of the larger ones (Table 2). I did this by crossing females carrying the deficiency chromosome over a balancer with males carrying the constitutively active calcineurin. If the daughter flies carrying the deficiency and *cnA-act* were as sterile as when they carried just *cnA-act* alone, I was able to eliminate that chromosomal region since it did not contain a gene that interacts with calcineurin. If sterility was suppressed in the daughters, I proceeded to test genes within that smaller deficiency. This enabled me to eliminate hundreds of genes under each larger deficiency, leaving me with approximately 10 to test for each one (Figure 6).

Deficiency *Df(2R)14H10Y-53* on the second chromosome was found to restore fertility to 45.9% (T. Aigaki, personal communication). After testing the smaller deficiencies, *Df(2R)Exel7149* and *Df(2R)BSC347* were both found to significantly suppress sterility ($P=0.0002$, $P=0.0012$; Figure 7; Table 2). The region where these two deletions overlap contains 11 genes and was considered to be the likely location of the candidate gene (Figure 6). Out of these 11 genes, *eIF3-S8* was found to significantly restore fertility of *cnA-act* females ($P=0.0013$) (Figure 8). The other 10 were not found

to significantly suppress sterility or significantly enhanced it, so they will not be discussed further (Table 1).

Deficiency *Df(2R)X58-12*, also on the second chromosome, was found to restore fertility to 19.4% (T. Aigaki, personal communication). After screening smaller deficiencies within it, *Df(2R)Exel7173* and *Df(2R)BSC598* were found to significantly suppress sterility (P=0.009, P=0.0104; Figure 9; Table 2). However, *Df(2R)Exel7173* is found within the slightly larger deletion *Df(2R)BSC597*, which showed no significant suppression (P=0.1046; Figure 9). Therefore, genes were not examined under this region. Overall, I narrowed down a region of 22 gene candidates under *Df(2R)BSC598*, 12 of which had mutant stocks available to be ordered. So far, 9 have been tested and 2 have been found to be potential candidate genes. Preliminary data from *RpS24* suggests that it significantly restores fertility of *cnA-act* females (P=0.0078; Figure 10). However, only 3 females were used for this fertility/fecundity test so additional tests must be done to confirm this result. *CG42565* was also found to suppress sterility (P<0.0001; Figure 11). The other 7 either did not suppress sterility or significantly enhanced it, so they will not be discussed further (Table 1).

For *Df(3L)AC1*, the smaller deletions *Df(3L)BSC390*, *Df(3L)BSC393*, *Df(3L)BSC576*, and *Df(3L)BSC283* were all found either to not suppress or to enhance the sterility phenotype (Figure 12; Table 2). However, there are still two smaller deletions that need to be tested in order to narrow down the region of candidate genes even further. Similarly, for *Df(2R)stan1*, the smaller deletions *Df(2R)BSC281*, *Df(2R)ED2098*, and *Df(2R)BSC336* all showed either no suppression or an enhancement of the sterility phenotype (Figure 13; Table 2). *Df(2R)BSC231* showed near-significant

suppression (P=0.020; Figure 13). However, there is still another smaller deletion that needs to be tested so genes under *Df(2R)stan1* have not yet been examined more closely.

DISCUSSION

The intricacies of the egg activation pathway are complex and elusive. This is due to the fact that it is such a rapid process that involves a vast number of changes within the egg. To dissect this pathway, I studied the genetic interactions between calcineurin and certain genes of interest that were uncovered by deletions known to suppress sterility in *cnA-act* flies. Genes whose heterozygous P-element insertions resulted in significantly increased hatchability of *cnA-act* progeny in a fertility/fecundity assay were determined to be candidate genes for the egg activation pathway. As stated previously, it was initially believed that the most likely candidate gene would be another phosphatase, a kinase or kinase binding protein. However, after performing fertility/fecundity assays on genes of this category under the first deficiency, *Df(1)JC70*, none of the ones tested were found to restore fertility to similar levels, so the rest of the genes under this deficiency were examined (Table 2).

CG6927

Mutant alleles of *CG6927*, a gene that is found under *Df(1)JC70*, showed the strongest suppression of *cnA-act* sterility. This gene currently has no known function and has not yet been studied in *Drosophila*. The sequence suggests that it may function as a gated ion channel (but the only evidence for this is sequence or structure similarity with other proteins involved with ion transfer; FlyBase). *CG6927* mutant heterozygosity

restored fertility of *cnA-act* females to nearly 100%, which is much higher than that seen for *Df(1)JC70* (Figure 4). This difference between the hatchability ratio for *CG6927* mutants and flies carrying *Df(1)JC70* could be due to the fact that haplo-insufficiency of other genes deleted by the deficiency somehow lowers the fertility of the female at the same time that haplo-insufficiency for *CG6927* raises it. Consistent with this idea, eggs of the *cnA-act* females have an average hatchability of 0.06. However, in many of the fertility/fecundity assays, eggs from the heterozygous mutants showed an enhanced sterility phenotype with a decrease in hatchability to nearly 0 (Figure 4). This could explain the difference between the hatchability values since there could be genes under the deficiency that actually enhance the sterility phenotype, which counteracts the sterility suppression of *CG6927*.

EIF3-S8

eIF3-S8 was found to be one gene under *Df(2R)14H10Y-53* whose mutant alleles suppressed the sterility of *cnA-act*. Based on sequence and structural similarity, this gene likely encodes a translation initiation factor (Flybase). Since there is a shift in which mRNAs get translated and which get degraded at egg activation (Tadros et al., 2003), it was exciting to find a translation factor as a possible downstream target of the egg activation signal. Translation initiation factors can be regulated by covalent modification via the addition or removal of a phosphate group (Morley & Pain, 1995). My results suggest that *eIF3-S8* may be downstream of calcineurin in the egg activation pathway, perhaps providing a link between the calcium signal for activation and translational control.

RPS24 AND CG42565

Mutations in two genes under *Df(2R)X58-12*, *RpS24* and *CG42565*, independently suppressed *cnA-act* sterility. Like *eIF3-S8*, *RpS24* is involved in translation, although additional experiments need to be performed in order to confirm its exact role (FlyBase; Brogna et al., 2002). However, since only 3 females were tested, the fertility/fecundity assay for this mutant, while encouraging (Figure 10) is very preliminary, and must be repeated with a larger sample size. *CG42565* has no known function in *Drosophila* and has not been examined for sequence or structural similarity. However, mutants in this gene significantly suppressed sterility of *cnA-act* females so it is a likely candidate gene for the egg activation pathway ($P<0.0001$; Figure 11). If results are reproducible for both genes, they should be examined in greater depth in order to determine the genes' precise roles in egg activation.

CONCLUSION AND FUTURE DIRECTIONS

These results confirm that *CG6927*, *eIF3-S8*, *CG42565*, and perhaps *RpS24* are candidates for involvement in the egg activation pathway due to their genetic interaction with calcineurin and 46 genes can be eliminated as candidates, including a number of phosphatases, kinases, and kinase-binding proteins. However, more study is needed to determine the other components of the pathway that are located within the additional deletions reported to suppress sterility. In addition, these candidate proteins must be examined for their exact roles and locations within the pathway and their specific effects during egg activation. To do so, it would be helpful to look at whether or not candidate

gene mutation homozygotes have any problems with egg activation or fertility in general (if they are not homozygous lethal).

In the future, it needs to be confirmed that all of these (especially the translation factor) do, in fact, interact with calcineurin and are not merely affecting the transgene construct that results in the constitutive calcineurin activity (Takeo et al., 2006). This can be tested by RT-PCR and Western blot. *RpS24* and *CG42565* must also be examined more closely to ensure that the result was not due to a limited sample size but is a true effect.

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REFERENCES

Allis, C., Waring, G., & Mahowald, A. (1977). Mass isolation of pole cells from Drosophila melanogaster. *Developmental Biology,* **56**, 372-81.

Backs, J., Stein, P., Backs, T., Duncan, F., Grueter, C., McNally, J., et al. (2010). The γ isoform of CaM kinase II controls mouse egg activation by regulating cell cycle resumption. *Proc Natl Acad Sci USA,* **107**(1), 81-6.

Brogna, S., Sato, T., & Rosbash, M. (2002). Ribosome components are associated with sites of transcription. *Molecular Cell*, **10**(1), 93-104.

Doane, W. (1960). Completion of meiosis in uninseminated eggs of Drosophila melanogaster. *Science,* **132**, 677-8.

Heifetz, Y., Yu, J., & Wolfner, M. (2001). Ovulation triggers activation of Drosophila oocytes. *Developmental Biology,* **234**, 416-24.

Horner, V. & Wolfner, M. (2008a). Mechanical stimulation by osmotic and hydrostatic pressure activates Drosophila oocytes in vitro in a calcium-dependent manner. *Developmental Biology,* **316**(1), 100-9.

Horner, V. & Wolfner, M. (2008b). Transitioning from egg to embryo: trigges and mechanisms of egg activation. *Developmental Dynamics,* **237**, 527-44.

Horner, V., Czank, A., Williams, B., Puro, J., Kubli, E., Wolfner, M., et al. (2006). Calcium regulation by the Drosophila calcipressin homolog, sarah, is required for several aspects of egg activation. *Current Biology,* **16**, 1441-6.

King, R. (1970). The meiotic behavior of the Drosophila oocyte. *International Review of Cytology,* **28**, 125-68.

Klee, C., Ren, H., & Wang, X. (1998). Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *Journal of Biological Chemistry,* **273**, 13367-70.

Liu, J. & Maller, J. (2005). Calcium elevation at fertilization coordinates phosphorylation of XErp1/Emi2 by Plx1 and CamKII to release metaphase arrest by cytostatic factor. *Current Biology,* **15**, 1458-68.

Mochida, S. & Hunt, T. (2007). Calcineurin is required to release *Xenopus* egg extracts from meiotic M phase. *Nature*, **449**, 336-41.

Morley, S., & Pain, V. (1995). Translational regulation during activation of porcine peripheral blood lymphocytes: association and phosphorylation of the alpha and gamma subunits of the initiation factor complex eIF-4F. *Biochemical Journal,* **312**, 627-35.

Nishiyama, T., Yoshizaki, N., Kishimoto, T., & Ohsumi, K. (2007). Transient activation of calcineurin is essential to initiate embryonic development in Xenopus laevis. *Nature,* **449**, 341-5.

Rauh, N., Schmidt, A., Bormann, J., Nigg, E., & Mayer, T. (2005). Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation. *Nature,* **437**, 1048-52.

Reber, S., Over, S., Kronja, I., & Gruss, O. (2008). CaM kinase II initiates meiostic spindle depolymerization independently of APC/C activation. *Journal of Cell Biology,* **183**(6), 1007-17.

Stricker, S. (1999). Comparative biology of calcium signaliing during fertilization and egg activation in animals. *Developmental Biology,* **211**, 157-76.

Tadros, W., Houston, S., Bashirullah, A., Cooperstock, R., Semotok, J., Reed, B., et al. (2003). Regulation of maternal transcript destabilization during egg activation in Drosophila. *Genetics,* **164**, 989-1001.

Takeo, S., Hawley, R., & Aigaki, T. (2010). Calcineurin and its regulation by Sra/RCAN is required for completion of meiosis in Drosophila. *Developmental Biology,* **344**(2), 957-67.

Takeo, S., Tsuda, M., Akahori, S., Matsuo, T., & Aigaki, T. (2006). The calcineurin regulator sra plays an essential role in female meiosis in Drosophila. *Current Biology,* **16**(14), 1435-40.

Weil, T., Parton, R., Davis, I., & Gavis, E. (2008). Changes in bicoid mRNA anchoring highlight conserved mechanisms during the oocyte-to-embryo transition. *Current Biology,* **18**(14), 1055-61.

Table 1. Candidate genes tested. * denotes those whose mutants actually showed significant enhancement of the sterility phenotype. These genes were not examined further. ** denotes genes whose mutants significantly suppressed the sterility phenotype.

Deletion	Gene	Position	n	Hatchability	P-value
Df(1)JC70	*CG3009*	4C11-4C11	9	0.108	0.086
	rap	4C11-4C12	9	0.005	0.006*
	Pp2C1	4C12-4C13	7	0.067	0.182
	ctp	4C13-4C14	10	0.001	0.002*
	CG7010	4C14-4C14	7	0.124	0.135
	CG7024	4C14-4C14	10	0.001	0.018*
	CG6986	4C14-4C15	8	0.000	0.002*
	CG2861	4D1-4D2	10	0.000	0.001*
	CG6927	**4D4-4D5**	**23**	**0.924**	**<0.0001****
	CG32772	4D5-4D5	10	0.048	0.612
	CG42594	4D5-4D5	10	0.005	0.219
	CG6903	4D6-4D6	8	0.044	0.627
	CG4068	4D6-4D7	12	0.118	0.883
	Ptp4E	4D7-4D7	6	0.013	0.128
	CG32767	4E2-4E2	8	0.018	0.193
	ovo	4E2-4E2	9	0.038	0.257
	rg	4E2-4F3	7	0.000	0.004*
	cdk7	4F4-4F4	3	0.005	0.130
	CanB	4F5-4F5	8	0.079	0.154
	sans fille	4F4-4F4	17	0.104	0.366
	CG4198	4F4-4F5	10	0.032	0.336
	XRCC1	4F5-4F5	8	0.016	0.230
	CG15930	4F5-4F5	10	0.003	0.003*
	sk	4F5-4F9	10	0.000	0.002*
	NAAT1	4F9-4F9	9	0.000	0.001*
	yu	4F9-4F10	6	0.059	0.581
	CG15784	4F10-4F10	10	0.053	0.709
	CG4165	4F10-4F10	10	0.001	0.037*
	CG12730	5A2-5A2	15	0.093	0.695
	Vsx2	5A3-5A5	7	0.000	0.007*
Df(2R)14H10Y-53	*CG10936*	54D1-54D2	8	0.079	0.678
	rhi	54D2-54D2	7	0.025	0.283
	CG18186	54D2-54D2	8	0.000	0.003*
	CG30106	54D3-54D3	9	0.037	0.351
	eIF3-S8	**54D4-54D4**	**10**	**0.187**	**0.007****
	CG30108	54D4-54D4	8	0.042	1.000

	CG30109	54D4-54D4	8	0.081	0.116
	CG6459	54D4-54D4	6	0.049	0.707
	sema-1B	54D4-54D5	10	0.005	0.098
	HPS4	54D5-54D5	10	0.061	0.367
	swi2	54D5-54D6	10	0.093	0.345
Df(2R)X58-12	*CG3927*	58F1-58F1	12	0.022	0.086
	CG34445	58F3-58F3	10	0.091	0.384
	RpS24	**58F3-58F3**	**3**	**0.630**	**<0.0001****
	Ugt58Fa	58F3-58F3	9	0.021	0.134
	CG2852	58F3-58F3	9	0.119	0.490
	CG13510	58F4-58F4	9	0.013	0.044*
	CG42565	**58F4-58F4**	**11**	**0.630**	**<0.0001****
	CG42566	58F4-58F4	10	0.005	0.006*
	CG3746	58F4-58F4	11	0.006	0.008*

Table 2. Smaller deletions within larger ones tested * denotes those that actually showed significant enhancement of the sterility phenotype. These regions were not examined further. ** denotes regions that significantly suppressed the sterility phenotype.

Deletion	Sub Deletion	n	Hatchability	P-value
Df(2R)14H10Y-53	***Df(2R)Exel7149***	**8**	**0.644**	**0.0002****
	Df(2R)BSC347	**9**	**0.524**	**0.0012****
	Df(2R)BSC348	10	0.024	0.4474
Df(2R)X58-12	*Df(2R)BSC597*	9	0.024	0.1046
	Df(2R)Exel7171	10	0.036	0.8237
	Df(2R)Exel7173	**10**	**0.145**	**0.009****
	Df(2R)BSC598	**10**	**0.182**	**0.0104****
Df(3L)AC1	*Df(3L)BSC390*	10	0.002	0.0127*
	Df(3L)BSC393	8	0.002	0.0148*
	Df(3L)BSC576	10	0.002	0.0076*
	Df(3L)BSC283	9	0.094	0.4474
Df(2R)stan1	*Df(2R)BSC281*	10	0.074	0.5281
	Df(2R)ED2098	6	0	0.0223*
	Df(2R)BSC336	9	0.028	0.5694
	Df(2R)BSC231	**10**	**0.14**	**0.0196****

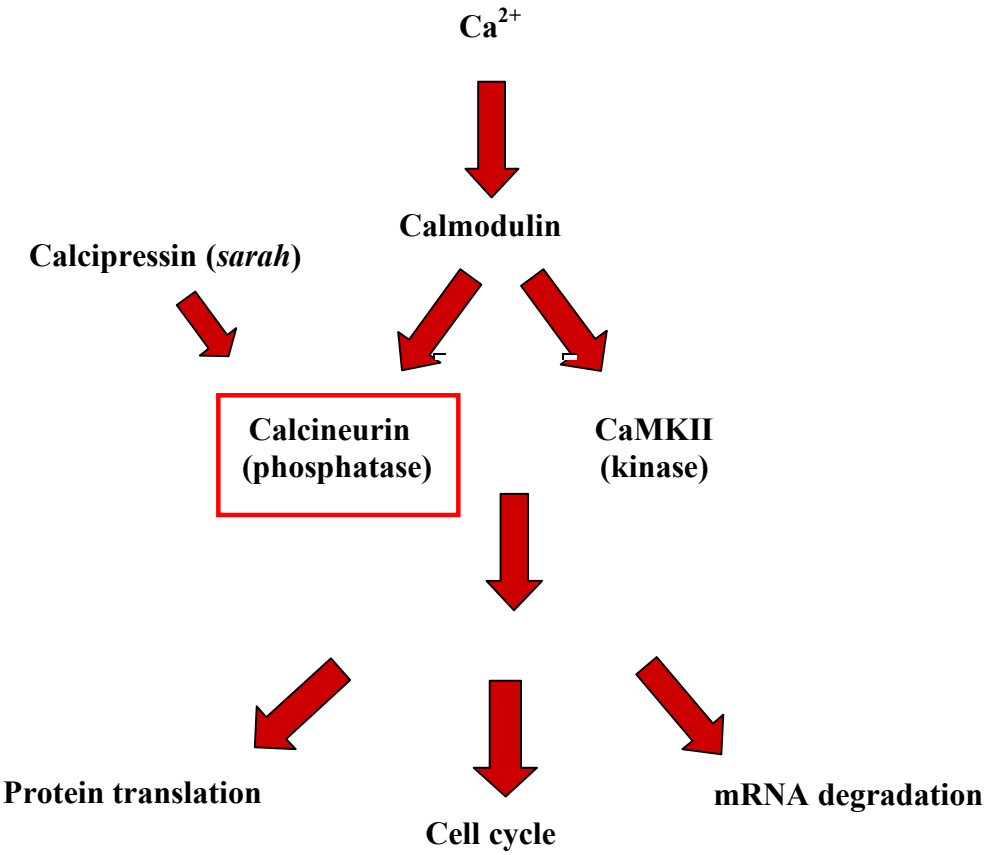

Figure 1. Egg activation pathway. A surge of calcium ions activates *calmodulin* which in turn activates *calcineurin* and *CaMKII*. This sets off a signal cascade that eventually leads to the key processes in egg activation (Horner & Wolfner, 2008a).

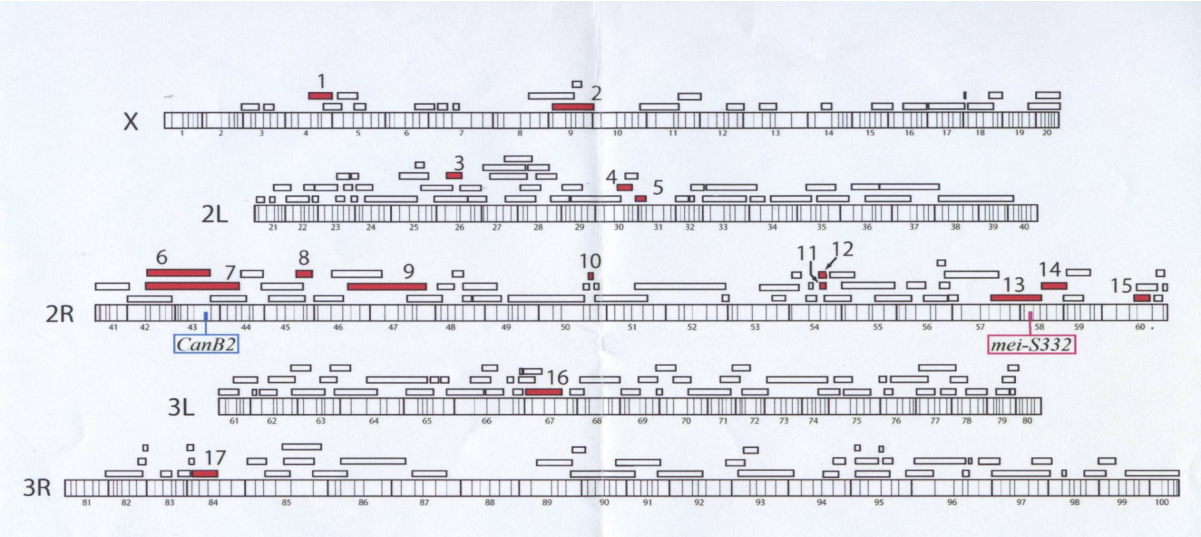

Figure 2. Each strip represents a part of the *Drosophila* genome. Bars above represent deletions tested by the Aigaki lab. Bars filled in red are deletions found to suppress sterility. Deletions 1, 9, 12, 14, 16 correspond to *Df(1)JC70, Df(2R)stan1, Df(2R)14H10Y-53 , Df(2R)X58-12*, and *Df(3L)AC1* and are the deletions examined in this paper (T. Aigaki, personal communication).

♂ $\frac{+}{Y}$; $\frac{nos>cnA\text{-}act}{Sb\ Ser}$ x $\frac{\text{Candidate gene mutant}}{Sb\ Ser}$; $\frac{+}{+}$ ♀

$\frac{\text{Candidate gene mutant}}{+}$; $\frac{+}{nos>cnA\text{-}act}$ ♀

Figure 3. Cross scheme to acquire flies carrying both the C-terminal truncated form of *cnA-act* driven in the germline by *nos* and a mutation for the gene to be tested.

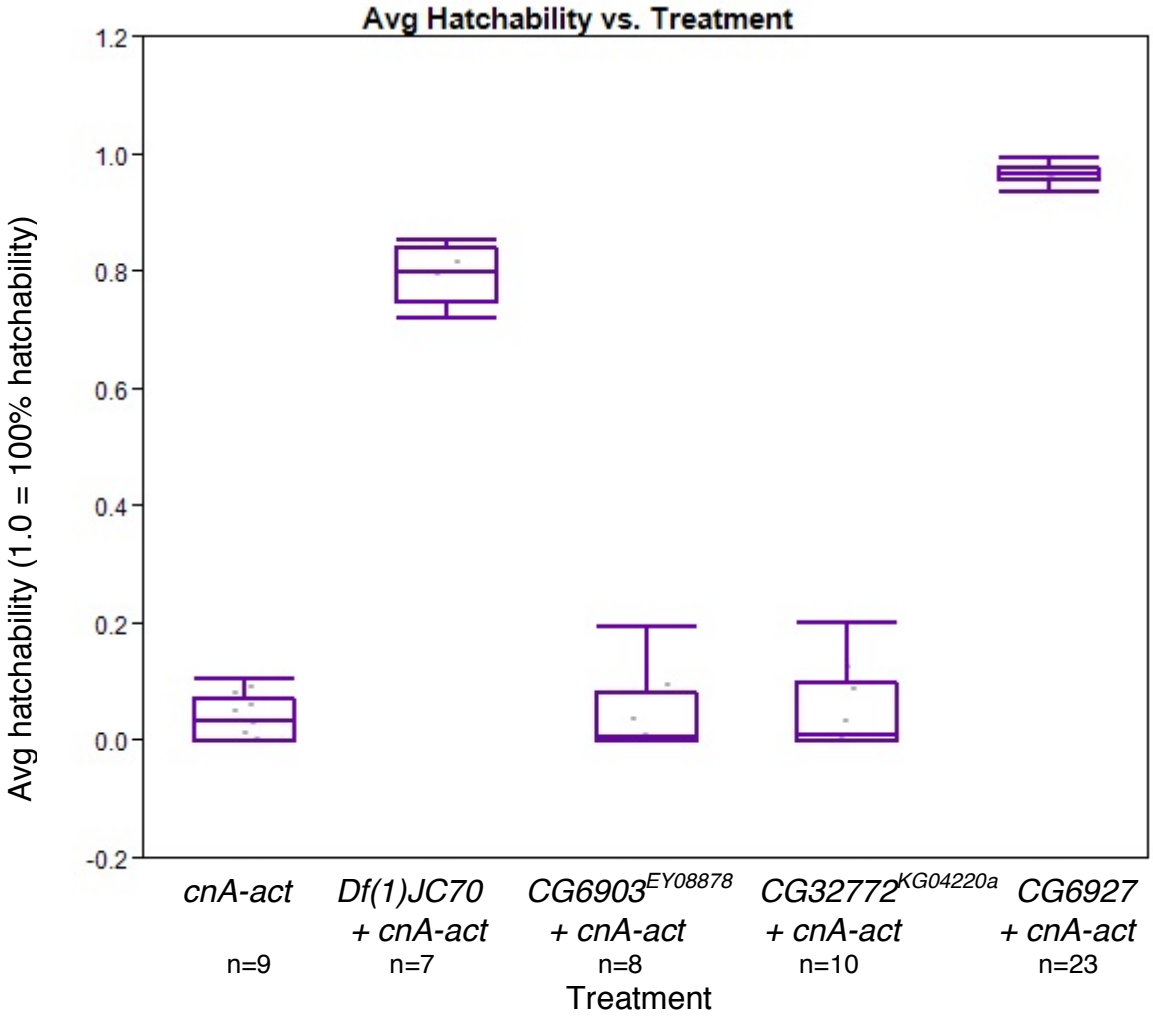

Figure 4. Fertility/fecundity assay for the first deletion: *Df(1)JC70* (P=0.0002), and three genes under this deficiency – *CG6903* (P=0.556), *CG32772* (P=0.612), *CG6927* (P<0.0001). *CnA-act* females were used for comparison. For box plots, center line represents the median. The line below designates the 1st quartile, and the line above is the 3rd quartile. The whiskers correspond to +/- 1½ of the Interquartile Range (IQR).

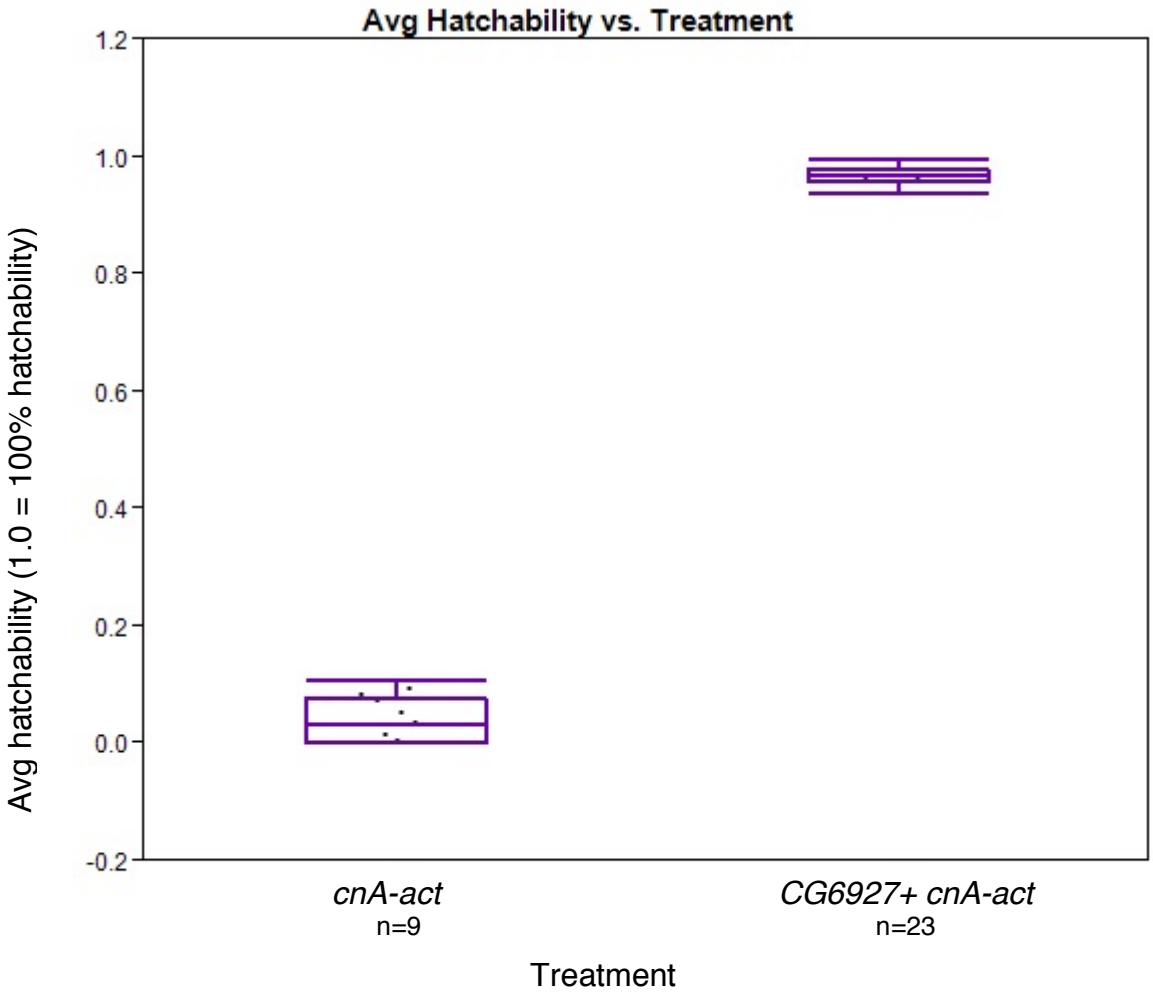

Figure 5. Fertility/fecundity assay for *CG6927*. A mutation in *CG6927* significantly suppressed sterility in *cnA-act* female flies (P<0.0001**). For box plots, center line represents the median. The line below designates the 1st quartile, and the line above is the 3rd quartile. The whiskers correspond to +/- 1½ of the Interquartile Range (IQR).

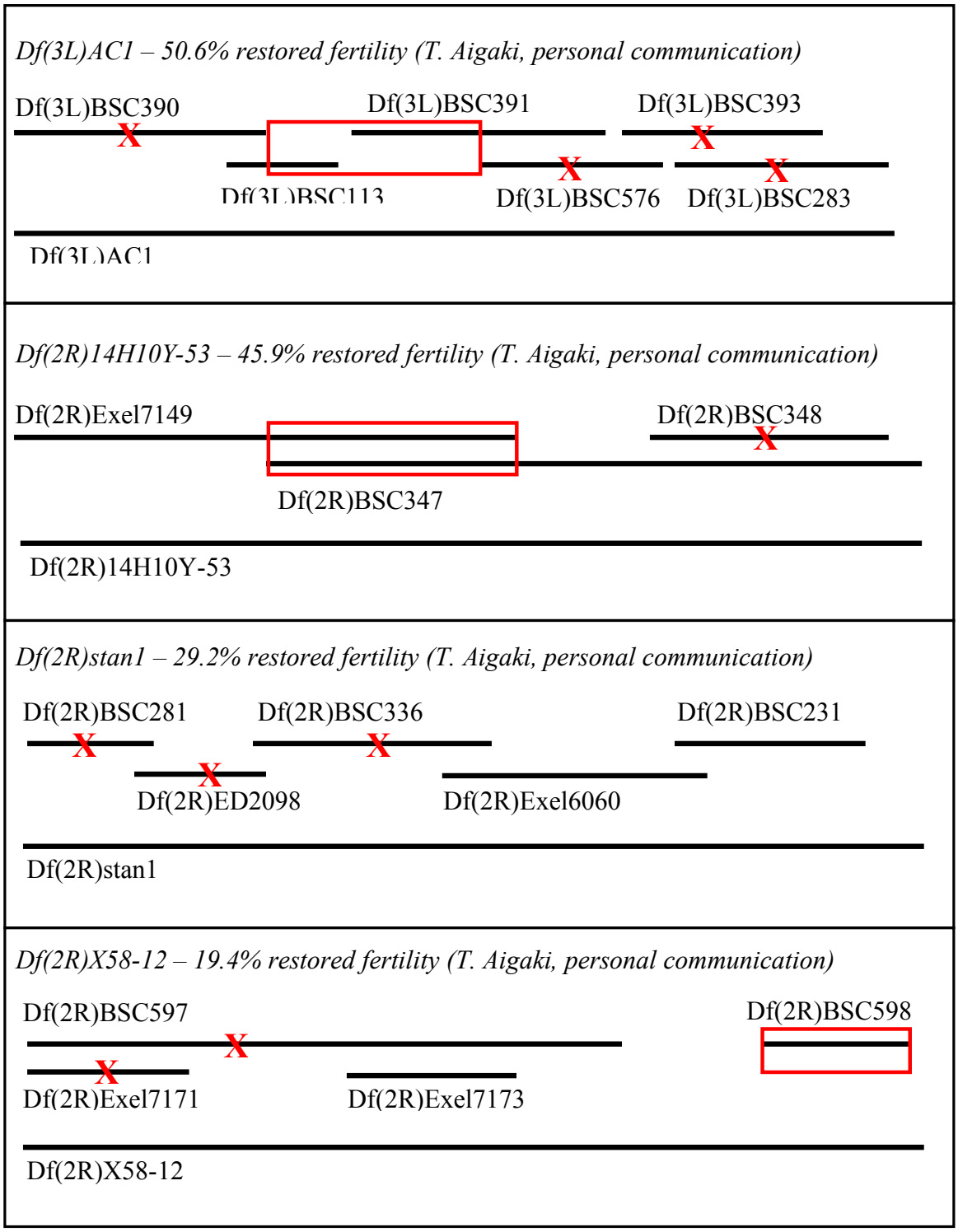

Figure 6. Four deletions examined (not including *Df(1)JC70*). Each deletion was broken up into smaller deletions that spanned the larger one. Red x's indicate regions that were ruled out. Red boxes indicate regions that are likely to contain a candidate gene for the egg activation pathway.

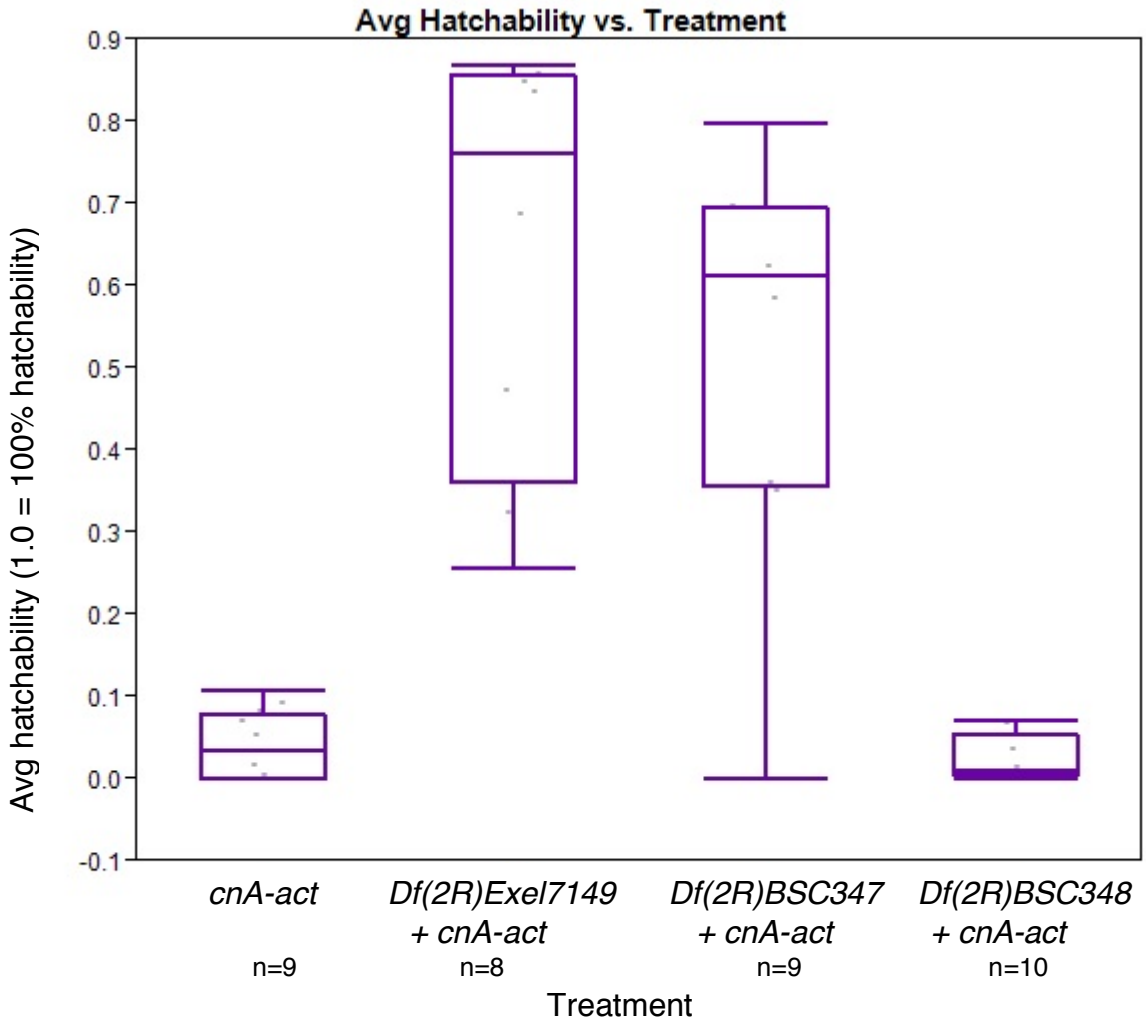

Figure 7. *Df(2R)14H10Y-53* screen for smaller deletions: *Df(2R)Exel7149* (P=0.0002**), *Df(2R)BSC347* (P=0.001**), *Df(2R)BSC348* (P=0.447). C*nA-act* females were tested for comparison. For box plots, center line represents the median. The line below designates the 1st quartile, and the line above is the 3rd quartile. The whiskers correspond to +/- 1½ of the Interquartile Range (IQR).

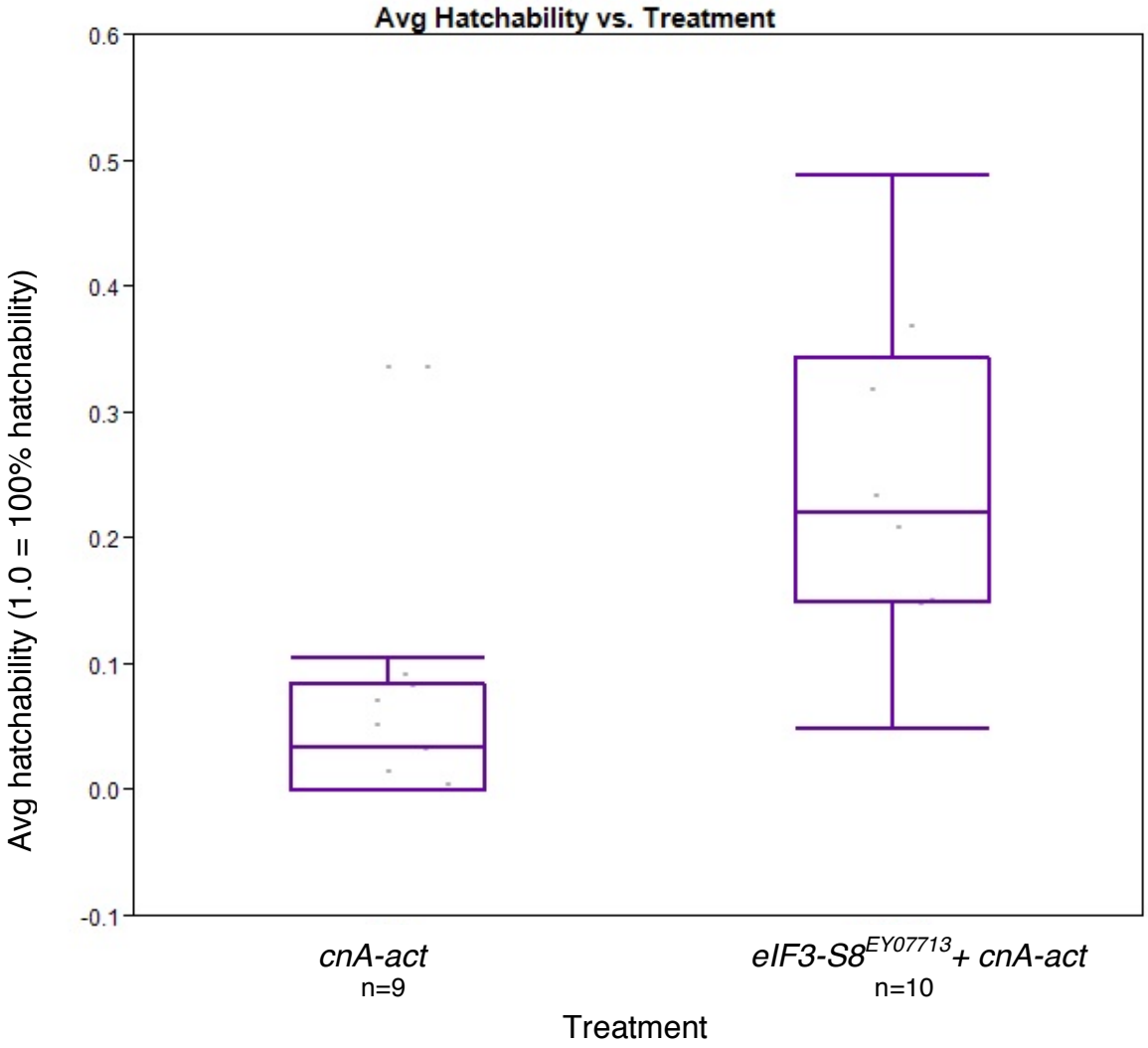

Figure 8. Fertility/fecundity assay for *eIF3-S8*. A mutation in *eIF3-S8* significantly suppressed sterility in *cnA-act* female flies (P=0.0013**). For box plots, center line represents the median. The line below designates the 1st quartile, and the line above is the 3rd quartile. The whiskers correspond to +/- 1½ of the Interquartile Range (IQR).

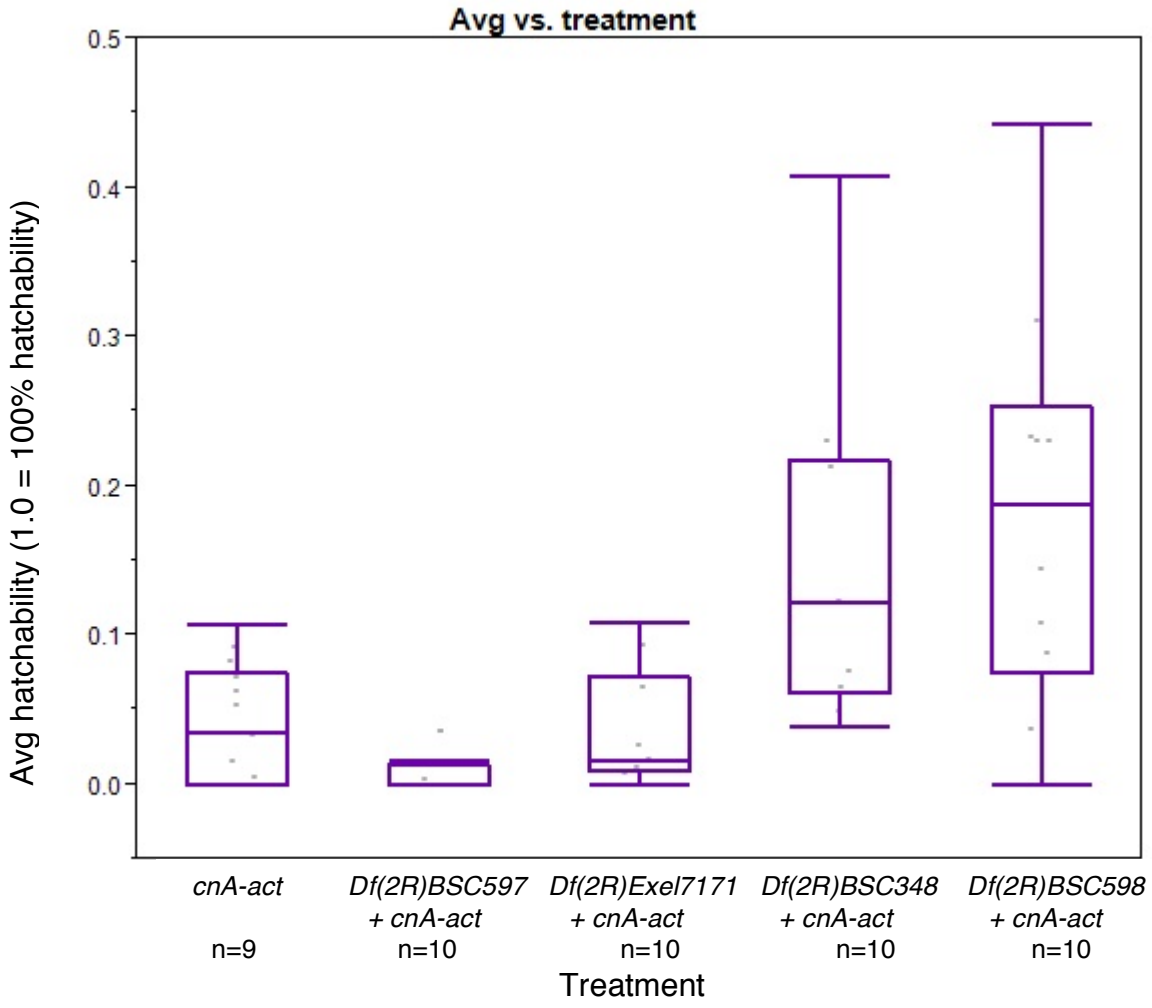

Figure 9. *Df(2R)X58-12* screen for smaller deletions: *Df(2R)BSC597* (P=0.105), *Df(2R)Exel7171* (P=0.824), *Df(2R)Exel7173* (P=0.009**), *Df(2R)BSC598* (P=0.010**). C*nA-act* females were tested for comparison. For box plots, center line represents the median. The line below designates the 1st quartile, and the line above is the 3rd quartile. The whiskers correspond to +/- 1½ of the Interquartile Range (IQR).

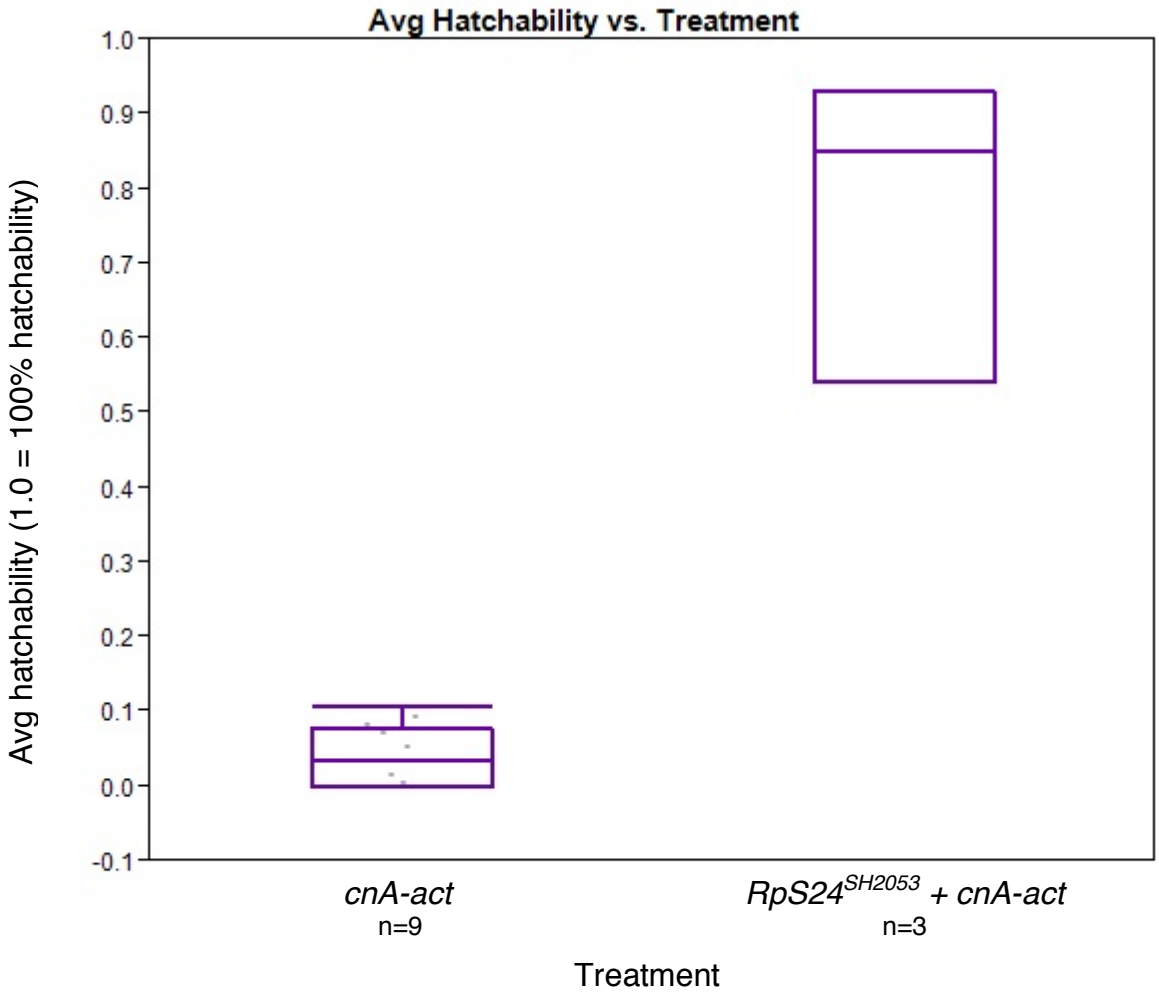

Figure 10. Fertility/fecundity assay for *RpS24*. A mutation in *RpS24* significantly suppressed sterility in *cnA-act* female flies (P=0.008**). *CnA-act* females were tested for comparison. For box plots, center line represents the median. The line below designates the 1st quartile, and the line above is the 3rd quartile. The whiskers correspond to +/- 1½ of the Interquartile Range (IQR).

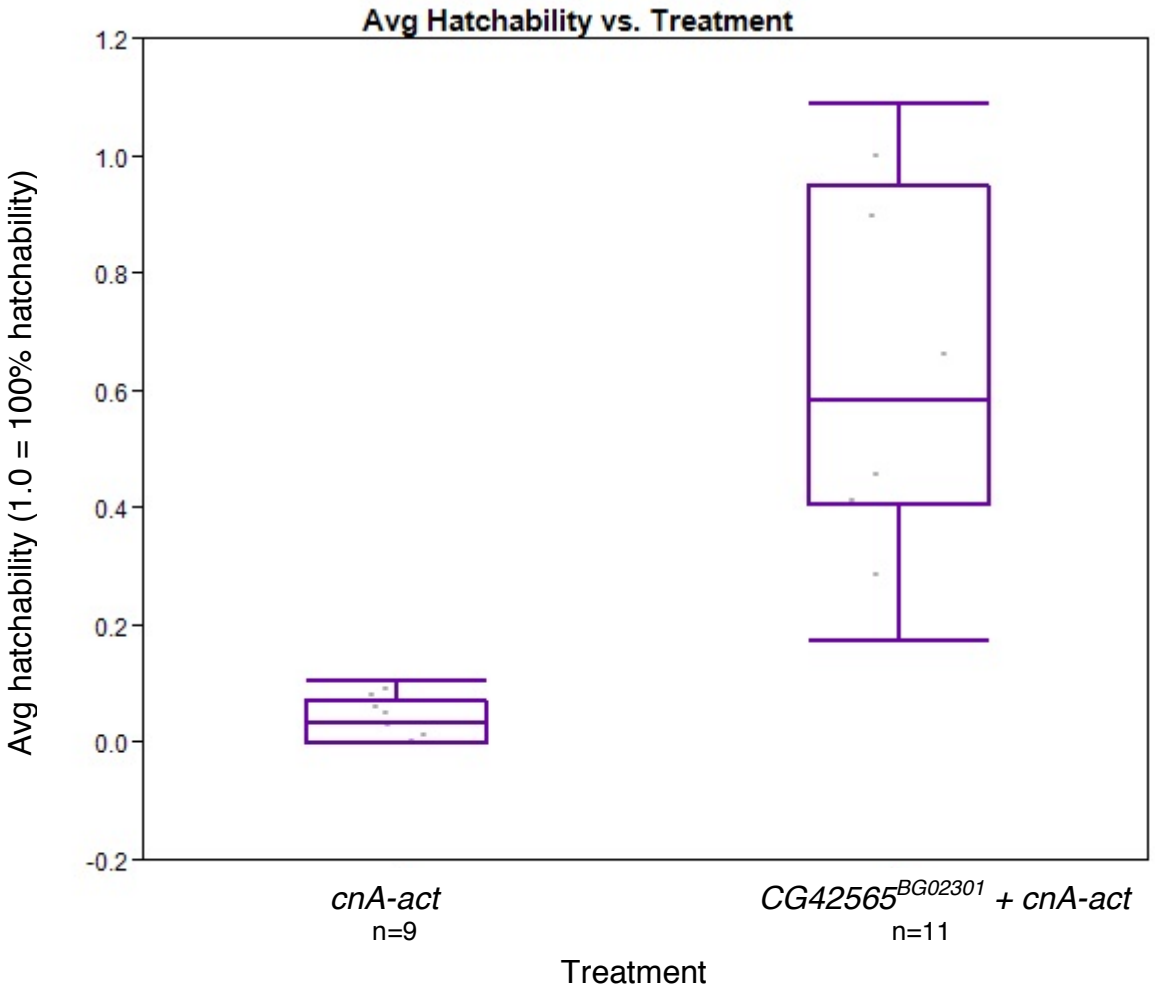

Figure 11. Fertility/fecundity assay for *CG42565*. A mutation in *CG42565* significantly suppressed sterility in *cnA-act* female flies (P<0.0001**). *CnA-act* females were tested for comparison. For box plots, center line represents the median. The line below designates the 1st quartile, and the line above is the 3rd quartile. The whiskers correspond to +/- 1½ of the Interquartile Range (IQR).

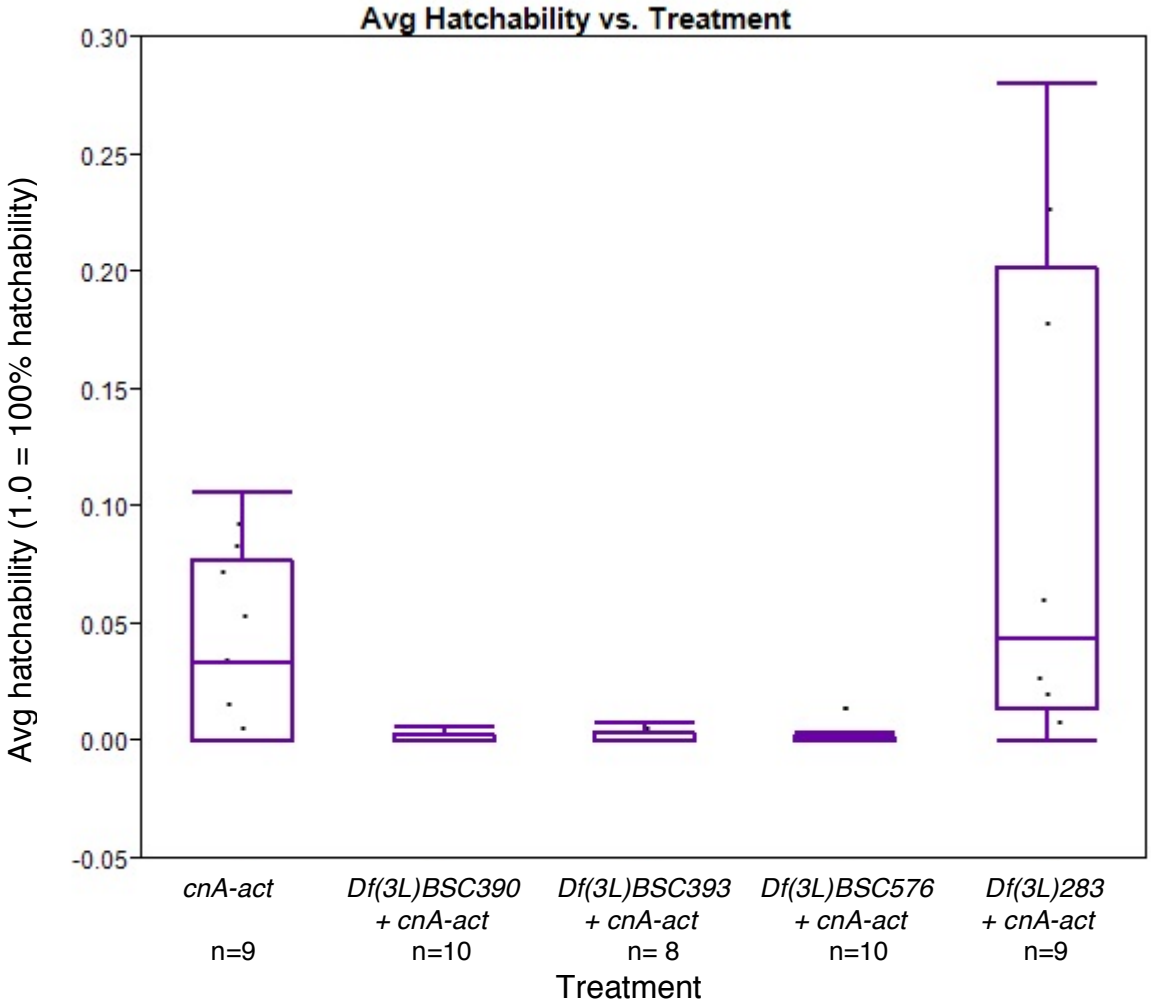

Figure 12. *Df(3L)AC1* screen for smaller deletions: *Df(3L)BSC390* (P=0.0127 – lower hatchability), *Df(3L)BSC393* (P=0.015 – lower hatchability), *Df(3L)BSC576* (P=0.008 – lower hatchability), *Df(3L)BSC283* (P=0.447). C*nA-act* females were tested for comparison. For box plots, center line represents the median. The line below designates the 1st quartile, and the line above is the 3rd quartile. The whiskers correspond to +/- 1½ of the Interquartile Range (IQR).

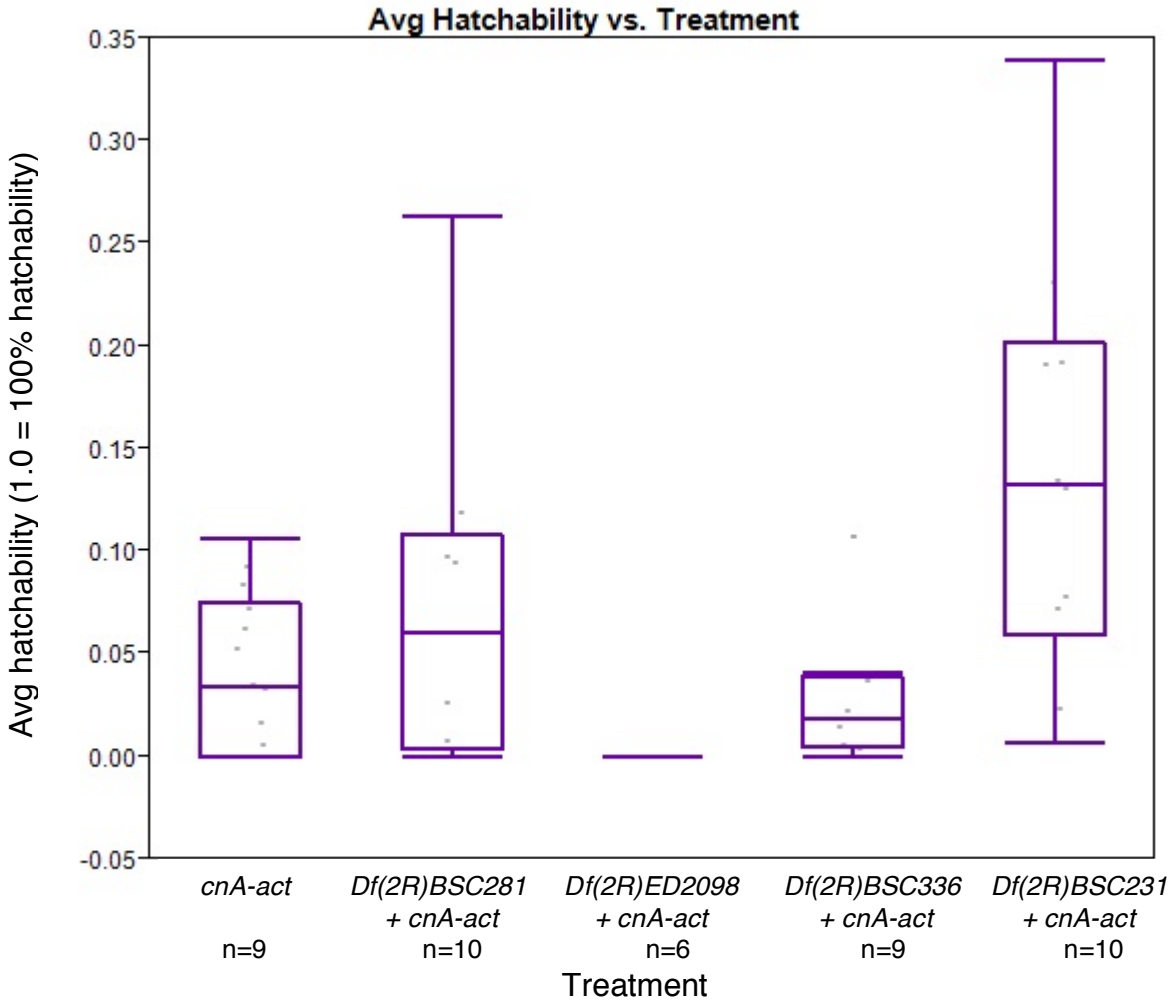

Figure 13. *Df(2R)stan1* screen for smaller deletions: *Df(2R)BSC281* (P=0.528), *Df(2R)ED2098* (P=0.022 – lower hatchability), *Df(2R)BSC336* (P=0.569), *Df(2R)BSC231* (P=0.020**). C*nA-act* females were tested for comparison. For box plots, center line represents the median. The line below designates the 1st quartile, and the line above is the 3rd quartile. The whiskers correspond to +/- 1½ of the Interquartile Range (IQR).